Biochemical and Biophysical Research Communications 382 (2009) 114–118

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Does domain swapping improve the stability of RNase A?

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ARTICLE INFO

Article history:
Received 15 February 2009
Available online 3 March 2009

Keywords:
Ribonuclease
RNase A
Domain swapping
Biomaterials

ABSTRACT

Self-assembling complexes have potential as novel supramolecular biomaterials but domain swapped complexes have yet to investigated in this capacity. Bovine ribonuclease A (RNase A) is a useful model protein as it is able to form a range of three dimensional domain swapped structures, including dimers, trimers and tetramers that have similar catalytic ability. However, little work has been carried out investigating the physical characteristics of these complexes. In an effort to characterise the strength of these oligomeric interactions, analytical ultracentrifugation was carried out to measure the dissociation of higher order complexes, using fluorescent tags to test for dissociation at very low concentrations. Results of this work suggest that the oligomers form a very tight complex, with no evidence of dissociation down to 250 pM. RNase A oligomers also had similar thermal stability to that of monomeric enzyme, suggesting that the main limiting factor in RNase A stability is the tertiary, rather than quaternary structure. Following thermal unfolding of RNase A, the protein refolded upon cooling, but returned to the monomeric state. This latter result may limit the potential of domain swapping as a means of material assembly.

Introduction

Higher order protein structures are of interest in the development of novel biomaterials [1,2], due to the range of naturally occurring proteins that have a wide range of properties. Of particular interest are protein fibres [3], which show great potential for applications including scaffolding, stabilisation and functionality. Molecular self-assembly of complexes is common in nature, and is now being used for producing novel supramolecular biomaterials [4]. Examples of self-assembling complexes include amyloid fibrils, a highly ordered form of protein that forms structured nanotubes [5] and three dimensional domain swapping, in which a bond is created between two or more protein molecules through the exchange of identical domains [6]. The potential of amyloid fibrils as biomaterials is well documented [7–9]; however, the potential of domain swapping as a means to assemble materials has yet to be explored.

Bovine pancreatic ribonuclease A (RNase A) is an example of a protein that can undergo domain swapping, in which two or more protein subunits exchange a common structural domain [10,11]. Domain swapped structures that have been observed by crystallography thus far include the C-dimer, formed by swapping the C-terminal domain [12], the N-dimer, formed by swapping the N-terminal domain [13], and the C-trimer, formed by each of three monomers swapping the C-terminal domain [14]. Other higher order aggregates, for which models have been proposed, include the NC-trimer, in which both the N-terminal and C-terminal domains swap [14], the NCN-tetramer and CNC-tetramer [6,15], pentamers [16], and also hexamers, heptamers, octamers, and nonamers [17].

Characterisation of the RNase aggregates reveal that all oligomers identified to date have catalytic activity. The relative activity of the different oligomers has been found to vary, depending on the assay used to measure enzyme activity. Assays using 2′,3′-cyclic cytidylate showed no difference in the specific activity of RNase A oligomers, while assays using polycytidylate, polyuridylate or yeast RNA as a substrate showed a slight decrease (40–90% of monomer activity) in specific activity with increasing oligomer size [16,18]. Conversely, assays using double-stranded RNase showed that the specific activity of RNase A increased with increasing oligomer size, with pentameric oligomers possessing a specific activity that is at least 10-fold greater than the monomer [16,18]. It is thought that the slight reduction in activity towards single-stranded RNA is due to reduced availability of the active sites to interact simultaneously with the RNA substrate, while the increased activity towards double-stranded RNA is due to an increase in the basic charge of the oligomers, which increases the ability of the enzyme to attack secondary structures [16,18]. Interestingly, the increased ability of higher order oligomers to attack double-stranded RNA is thought to be the reason underlying the increased antitumour action of RNase A oligomers [19,20].

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0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.02.142

One of the implications of the RNase A oligomeric structure is that it may provide information of how domain swapping of proteins may be involved in the formation of amyloid fibril formation [12,13]. Modification of the RNase A enzyme to incorporate an extended hinge loop resulted in an amyloid-like fibril, composing a cross-β spine with 3D domain swapped functional units that retained enzyme activity [21]. Of interest to us is the effect that changing the quaternary structure has on the stability of the enzyme. Since putative biomaterials assembled via a domain swapped mechanism retain active site domains, they represent ideal candidates for nanostructures incorporating active functionality. However, the physical strength of these structures is relatively unknown. RNase A provides a model protein to directly compare the stability of enzymes that have the same amino acid sequence, but different quaternary structures. Previous studies have suggested that dimeric RNase A is more stable than trimeric or tetrameric variants [15], but have not been studied in detail. Studies of a novel RNase A dimer composed of monomeric units covalently linked by a single amide bond showed little change in thermal stability [22].

In this study, we measure the thermal stability and dissociation of a range of domain swapped RNase A structures, with a view to testing their suitability for incorporation into self-assembled biomaterials.

Materials and methods

Preparation of RNase aggregates. Ribonuclease A from bovine pancreas (Type XII-A) was obtained from Sigma. RNase A aggregates were prepared as previously described [16], through lyophilisation of RNase A from a 40% acetic acid solution. As previously described, [16] the aggregates were separated by cation exchange chromatography using a Source 15S PE 4.6/100 column, and a gradient of 20–200 mM NaPi, pH 6.7.

Assay for enzymatic activity. Ribonuclease activity was measured using the RNase Alert fluorescent substrate (Ambion), as previously described [22]. Assays were carried out using a plate reader equipped with fluorescence optics, using an excitation filter of 485 nm and an emission filter of 520 nm. Typical assays contained 0.01 ng/mL (0.73 nM) of RNase A. The rate of formation of the product/s/mg was calculated from the initial slope of the graph, and the activity of the unmodified enzyme was set at 100%. The activities of the variants were reported relative to the activity of the unmodified enzyme. The negative control reaction contained the substrate with the assay buffer.

Fluorescent labelling of RNase A. The monomer, C-dimer and NC-trimer forms of RNase A (5–30 nmol) were desalted into buffer containing 100 mM $NaHCO_3$ (pH 8.0), and Alexa Fluor 488 C-5 maleimide dye (Invitrogen) was added in a 2- to 7-fold excess. The solution was incubated in the dark for 2 h, before the reaction was quenched by adding 20 mM Tris (pH 8.0) and the labelled protein was separated from unbound label by gel filtration, initially using a 5 mL HiTrap desalting column (GE Healthcare), before being loaded onto a Sephadex 200 10/300 column (GE Healthcare) and eluted with 20 mM Tris–HCl, 150 mM NaCl, pH 8.0 at 4 °C. Protein concentration and the degree of labelling were determined by measuring the absorbance at 280 nm and 494 nm. It was found that the protein was labelled with 0.8 mol dye/mol protein for the monomer, 1.2 mol dye/mol protein for the C-dimer and 2.2 mol dye/mol protein for the NC-trimer.

Analytical ultracentrifugation. Sedimentation velocity experiments were performed in a Beckman Coulter Model XL-A analytical ultracentrifuge equipped with UV/vis scanning optics or a fluorescence-detection system (Aviv Biomedical). Protein sample and reference (50 mM NaPi, 100 mM NaCl, pH 7.6) solutions were loaded into 12 mm double-sector cells with quartz windows or double-sector centrifuge cells with sapphire windows. Samples (380 μL) and reference solution (400 μL) were centrifuged at 55,000 or 50,000 rpm at 20 °C and data were collected in continuously. Data were fitted to a continuous size-distribution model using the program SEDFIT [23]. The partial specific volume ($\bar{\nu}$) of the sample (0.709 mL g^{-1}), buffer density (0.998 g mL^{-1}) and buffer viscosity (1.002 cp) were computed using the program SEDNTRP [24].

Differential scanning fluorimetry. Thermal shift assays were carried out as described previously [25]. Twenty-five microlitres of solution containing 0.5 mg/mL protein, 50 mM NaPi pH 6.7 and 10× Sypro Orange dye (Invitrogen) were added to the wells of a 96-well thin-wall PCR plate (Bio-Rad). The plates were sealed and heated in an iCycler iQ Real Time PCR Detection System (Bio-Rad) from 20 to 80 °C in increments of 0.2 °C, with 10 s dwell time. Fluorescence changes in the wells of the plate were monitored simultaneously with a charge-coupled device (CCD) camera. The wavelengths for excitation and emission were 490 and 575 nm, respectively. Experiments were carried out in triplicate for each condition.

Circular dichroism spectroscopy. CD spectroscopic data were generated using a Jasco J-815 circular dichroism spectrophotometer. Spectra were collected at a concentration of 0.03 mg/mL (2.3 μM) enzyme in buffer containing 20 mM Na_2HPO_4, pH 6.7. Temperature scans were monitored at 220 nm, and data were collected at 0.5 °C intervals between 40 and 70 °C with a 1 s averaging time. Cuvettes were stoppered during temperature scans to prevent evaporation.

Breakdown products of oligomers. RNase oligomers were separated by cation exchange chromatography, as described above, and were transferred to buffer containing 20 mM NaPi, pH 6.7. Samples (250 μL, 0.5 mg/mL) were loaded onto a Source 15S PE 4.6/100 column, and eluted with a gradient of 20–200 mM NaPi, pH 6.7 to confirm the oligomeric nature of the aggregates. Samples were then heated at 60 °C for 2 min before being cooled on ice and applied to the column.

Results and discussion

Preparation and activity of RNase aggregates

Based on the methods of Gotte et al. [16] RNase A was lyophilised from 40% acetic acid solutions, and separated by cation exchange chromatography (Fig. 1). The different oligomers were subsequently purified further by repeating the cation exchange chromatography step. As previously observed [16], the mixture contained predominantly monomer protein, followed by the C-dimer, N-dimer, NC-trimer, and C-trimer. Smaller amounts of higher order species were also observed. A fluorescently labelled single-stranded RNA substrate was used to measure the activity of the

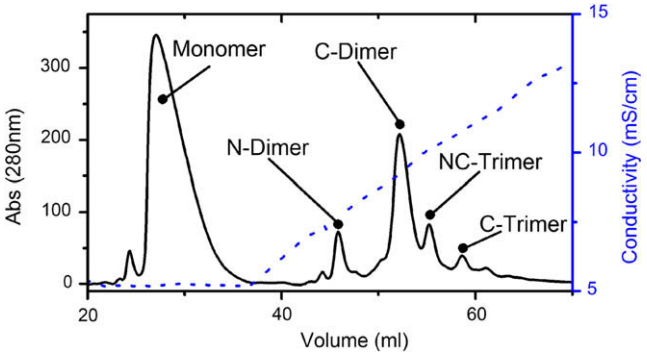

Fig. 1. Purification of RNase A oligomers by cation exchange chromatography.

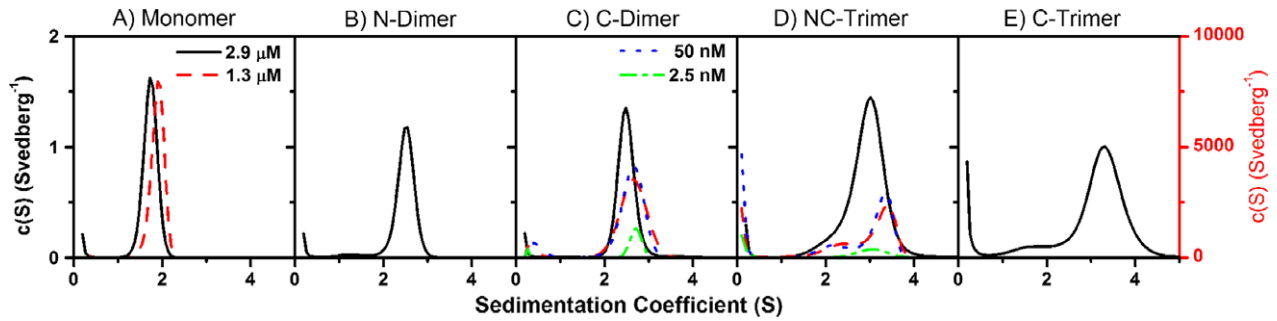

Fig. 2. Absorbance-detection (solid line, left ordinate) and fluorescence-detection sedimentation velocity experiments were carried out on oligomeric variants of RNase A. Absorbance-detection studies (broken lines, right ordinate) were carried out at 2.9 μM (90 μg/mL), while fluorescence-detection studies were carried out at 1.3 μM (solid lines), 50 nM (dashed lines), and 2.5 nM (dotted lines).

different oligomers by monitoring the increase in fluorescence over time [22]. This showed that each of the oligomers had very similar activity when expressed in units/pg of protein, similar to that previously observed for single-stranded RNA as a substrate [18].

Analytical ultracentrifugation

Analytical ultracentrifugation was used to confirm the oligomeric nature of the RNase A oligomers. Sedimentation velocity experiments showed that the RNase A species were homogenous in solution, with a single species observed for each preparation (Fig. 2A and Table 2). Monomeric RNase A had a sedimentation coefficient of 1.7S, and a molar mass of 15 kDa, which is similar to the expected mass of 13.7 kDa, with the higher order species also showing the expected sedimentation coefficients.

Table 1
Activity and stability of RNase A oligomers.

	Monomer	N-Dimer	C-Dimer	NC-Trimer	C-Trimer
% of total protein	63.2	5.9	19.9	6.5	4.5
Activity (U/pg)	0.10 ± 0.01	0.10 ± 0.01	0.09 ± 0.02	0.11 ± 0.02	0.10 ± 0.02
T_m (CD)	60.6 ± 0.3	60.7 ± 0.2	60.5 ± 0.2	60.7 ± 0.2	60.5 ± 0.2
T_m (DFS)	63.4 ± 0.1	63.4 ± 0.1	63.2 ± 0.1	63.6 ± 0.1	63.5 ± 0.1

Table 2
Properties of RNase A oligomers calculated by sedimentation velocity analyses.

		$s_{20,w}$[a]	f/f_o[b]
Monomer	2.9 μM	1.7	1.38
	1.3 μM	1.9	1.31
N-Dimer	2.9 μM	2.5	1.51
C-Dimer	2.9 μM	2.9	1.57
	1.3 μM	2.7	1.48
	50 nM	2.7	1.44
	2.5 nM	2.8	1.4[c]
	250 pM	2.8	1.4[c]
NC-Trimer	2.9 μM	3.0	1.60
	1.3 μM	3.3	1.42
	50 nM	3.3	1.44
	2.5 nM	3.1	1.43
	250 pM	3.0	1.3[c]
C-Trimer	2.9 μM	3.3	1.65

[a] Standardized sedimentation coefficient calculated from the ordinate maximum of each peak shown in the $c(s)$ distribution best fit (Fig. 2).
[b] Frictional ratio calculated from the vbar method [23].
[c] Frictional ratio was fixed for calculation of the sedimentation coefficient due to a high signal to noise ratio.

To probe the stability of the higher order species, fluorescence-detected sedimentation studies were used at low concentrations of enzyme. This technique involved using RNase oligomers labelled with the Alexa Fluor 488 succinimidyl ester, and allowed data to be collected down to 250 pM (8 ng/mL). At these low protein concentrations, analysis of the data was complicated by a high signal to noise ratio, necessitating the frictional ratio to be fixed

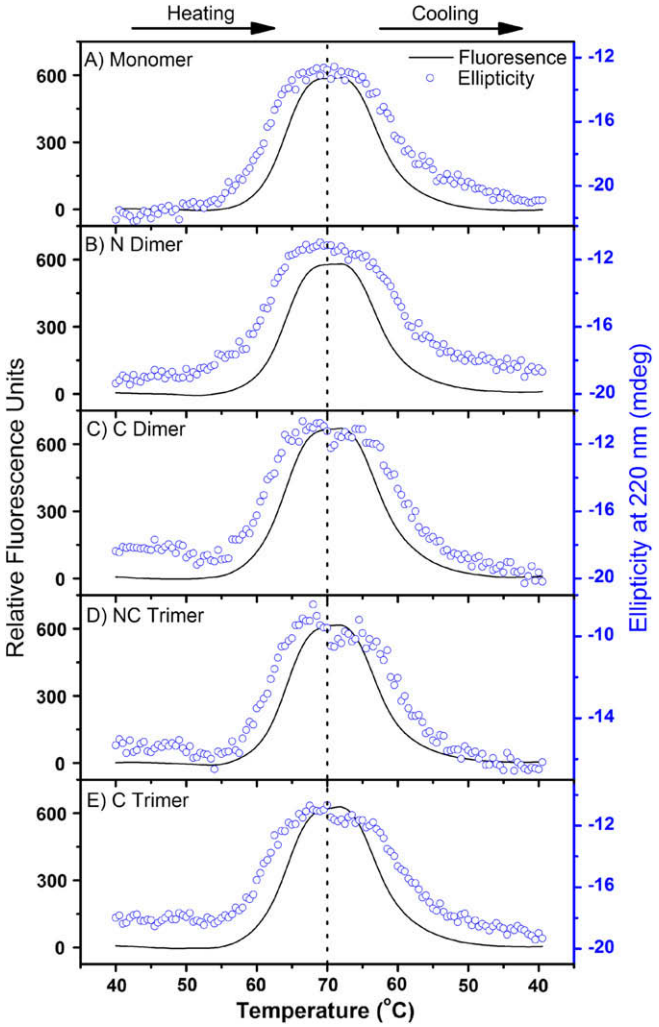

Fig. 3. CD spectroscopy (symbols, left ordinate) and differential scanning fluorimetry (lines, right ordinate) reveals that all RNase A variants had similar thermal stability. Samples were suspended in 20 mM NaPi buffer (pH 6.7) and incubated from 40 to 70 °C before being cooled back to 40 °C.

for calculation of the sedimentation coefficient. However, even at these low concentrations, the C-dimer and NC-trimer species showed no concentration dependent dissociation (Fig. 2B and Table 2), suggesting that the dissociation constant is substantially less than the nM range.

Thermal stability

Previous studies have suggested that oligomeric structure plays a role in protein stability, with many thermophilic enzymes having a higher oligomeric state than their mesophilic counterparts. Examples include phosphoribosylanthranilate isomerase, which is dimeric and stable in *Thermotoga maritima*, but monomeric and labile in mesophiles [26], ornithine carbamoyltransferase, which is dodecameric and stable in *Pyrococcus furiosis*, but trimeric in mesophiles [27], and malate dehydrogenase, in which the tetrameric enzyme from *Chloroflexus aurantiacus* is more stable than the dimeric enzyme from mesophiles [28].

Domain swapped oligomers of RNase A provide an opportunity to directly compare the thermal stability of proteins that have the same amino sequence, but different quaternary structures. RNase A has been the subject of extensive folding and refolding studies (reviewed in [29,30]), and is able to refold after thermal denaturation.

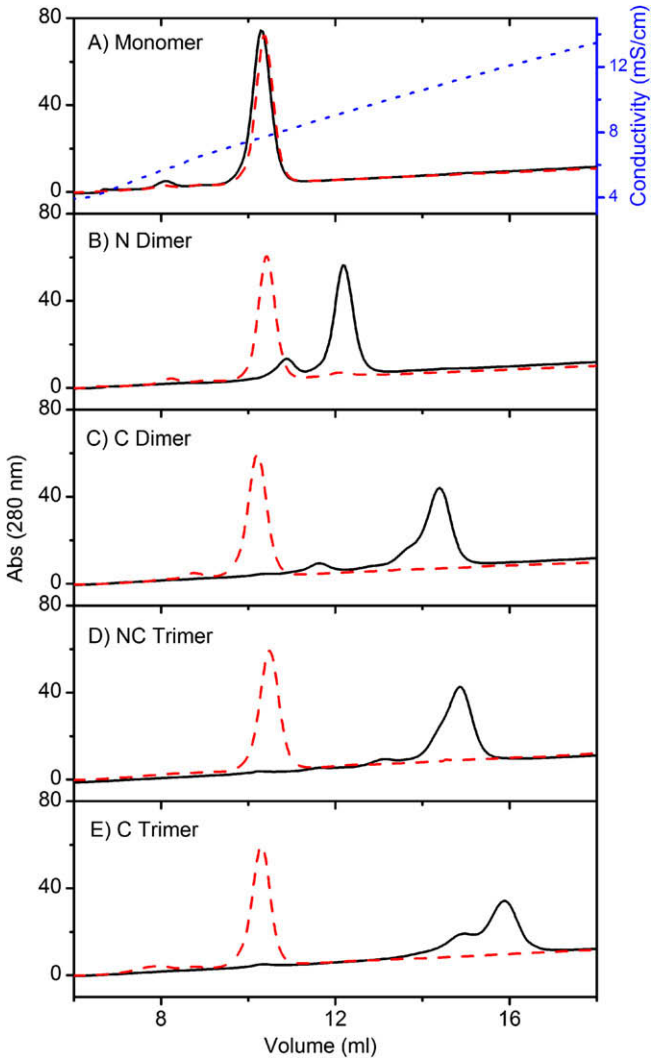

Fig. 4. Samples of RNase A oligomers (solid lines) were separated by cation exchange chromatography using a 20–200 mM NaPi gradient (indicated in panel A, right ordinate). Samples were then heated at 60 °C for 2 min before being applied to the column (dashed lines).

To determine the effect of domain swapping on thermal stability, CD spectroscopy and differential scanning fluorimetry were used to measure the melting temperatures of different RNase oligomers.

A CD spectrometer was used to monitor the ellipticity at 220 nm while the enzyme was incubated up to 70 °C. The CD spectrum of all the oligomers underwent a dramatic increase in ellipticity, with wavelengths scans consistent with unfolded protein. A sigmoidal curve fitted the data well and gave a melting temperature of around 60.5 °C for all variants (Fig. 3 and Table 1). Reducing the temperature back to 40 °C resulted in refolding of the enzyme, and gave a similar transition point.

To confirm these results, the assays were repeated using differential scanning fluorimetry, a technique that measures the thermal unfolding of proteins in the presence of a fluorescent dye. RNase oligomers were heated to 70 °C in the presence of Sypro Orange, a dye which is normally quenched in aqueous solutions but binds to the exposed hydrophobic interior regions of the unfolding protein, leading to an increase in fluorescence emission. All of the oligomers showed a similar increase in fluorescence upon heating, and a subsequent decrease in fluorescence upon cooling, with a transition point of around 63.4 °C for all variants (Fig. 3 and Table 1). These values agree well with the values obtained by CD spectroscopy.

Dissociation of aggregates

Previous studies had shown that the NC-trimer and C-trimer dissociate into dimers and monomers during long term storage, and after 5 min incubation at 55 °C [6]. In order to further investigate whether higher order quaternary structure is retained after melting and refolding, samples were incubated at 60 °C for 2 min, before the quaternary structure was determined by cation exchange chromatography. Following the brief incubation, all samples eluted at a position corresponding to the monomeric enzyme (Fig. 4), suggesting that the higher order quaternary structure had not been retained.

Conclusions

Domain swapping proteins shows potential as an *in vitro* assembly tool in the development of novel biomaterials due to their retention of catalytic activity, and low dissociation rates, as shown here using analytical ultracentrifugation. However, domain swapping does not increase the thermal stability of higher order aggregates, and higher order quaternary structure is lost at higher temperatures, placing limitations of the use of domain swapped structures as biomaterials. To confirm these observations, future work needs to be carried out on other domain swapped proteins, in order to determine the strength and stability of intersubunit interactions on systems less intrinsically stable than ribonuclease.

Acknowledgments

This work was funded, in part, by the Defence Threat Reduction Agency via the Army Research Office. We thank Jackie Healy for superlative technical support.

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